Thermal Resistance of Nonproteolytic Type B and Type E *Clostridium botulinum* Spores in Phosphate Buffer and Turkey Slurry†

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(MS# 94-157: Received 1 July 1994/Accepted 22 December 1994)

ABSTRACT

The heat resistance of nonproteolytic type B and type E *Clostridium botulinum* spores in phosphate buffer and turkey slurry was determined from 70 to 90°C. Thermal-death times were determined in vials heated using a water bath. Recovery of heat-injured spores was on reinforced clostridial medium (RCM) and tryptic soy agar (TSA) with and without added lysozyme (10 µg/ml). Decimal-reduction times (D-values) were determined by fitting a survival model to the data using a curve-fitting program. The apparent or measured heat resistance was maximum with RCM supplemented with lysozyme. The D-values at 80°C for type E spores in buffer ranged from 1.03 min for strain Whitefish to 4.51 min for strain Saratoga. The D-value for the most heat-resistant nonproteolytic type B strain KAP B5 in buffer was 4.31 min at 80°C. The z-values in buffer for all strains were very similar, ranging from 8.35 to 10.08°C. Turkey slurry offered protection to the spores with a concomitant increase in heat resistance. The D-values in turkey slurry ranged from 51.89 min at 70°C to 1.18 min at 85°C for type E strain Alaska (z = 9.90°C) and from 32.53 min at 75°C to 0.80 min at 90°C for nonproteolytic type B strain KAP B5 (z = 9.43°C). Thermal-death-time values from this study will assist food processors to design thermal processes that ensure safety against nonproteolytic *C. botulinum* in cook/chill foods.

Key words: *Clostridium botulinum*, spores, heat resistance, inactivation

Psychrotrophic strains of *Clostridium botulinum*, types E and F, and nonproteolytic type B, are recognized as a potential hazard in a new generation of minimally processed food products (*sous-vide* products and other refrigerated pasteurized foods of extended durability). Such products usually receive a mild heat treatment, contain no added preservatives, rely on refrigeration for extended shelf life, and are not reheated or are mildly heated prior to consumption. Spores of psychrotrophic *C. botulinum* possess sufficient heat resistance to allow them to survive pasteurization (cooking) treatments applied to such new minimally processed foods and to germinate, grow, and produce toxin at temperatures as low as 3.3°C *(12, 18, 24, 34)*. The food-safety implications of the increasing trend towards mild heat treatments aimed at retaining the optimal food characteristics need to be fully understood. While there has not been any reported case of botulism associated with minimally heated processed food products, changes in consumer life styles, the demand for meals requiring minimal preparation time, and growing utilization of ready-to-eat products in food-service operations increase the probability that an outbreak could occur. It is logical, therefore, that the heat treatment required to give a specified lethality for spores of psychrotrophic *C. botulinum* strains should be well defined.

Determination of spore survival after exposure to heat is characterized by the ability of the injured spores, which are more nutritionally demanding than unheated spores, to grow and form colonies on the recovery media. Sufficient evidence exists to document that the addition of lysozyme to the recovery medium resuscitates and increases the recovery of the heat-injured spores *(2, 5, 23, 28, 36, 37)*. In well-documented reviews on the recovery of injured spores, Roberts *(31)* and Adams *(1)* outlined the importance of the recovery media for injured spores and classified the additives which can increase recovery according to their mechanisms of action.

To our knowledge, there are no reported studies of the heat-resistance of nonproteolytic type B spores in foodstuffs. With type E spores, previous workers have conducted thermal inactivation studies primarily in foods of aquatic origin such as smoked fish *(9)*, ground whitefish chubs *(10)*, crab meat *(26)*, oyster homogenates *(6, 8)*, and menhaden surimi *(30)*. Data available that give a quantitative assessment of the thermal characteristics of the psychrotrophic strains of *C. botulinum* spores in meat products are insufficient to completely assess the importance of these organisms in the preservation of minimally processed refrigerated foods. The aim of the studies reported here was to provide information on the heat resistance of nonproteolytic *C. botulinum* spores (D- and z-

† *Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature.*

values) suspended in phosphate buffer and turkey slurry, to enable food processors to establish heat treatments suitable to inactivate these spores in minimally processed foods. The study also explored the possibility of predicting heat resistance in turkey from data obtained in buffer and evaluated two recovery media, with or without lysozyme, for the recovery of spores surviving heat treatment.

MATERIALS AND METHODS

Strains

Three nonproteolytic *C. botulinum* type B and three type E strains were used in the study. Nonproteolytic type B strains obtained from Campbell Soup Company, Camden, NJ, were CBW 25 and 17B. Nonproteolytic type B strain KAP B5, and type E strains Whitefish, Saratoga, and Alaska, were obtained from National Food Processors Association, Washington, D.C. The strains were grown in cooked-meat medium (Difco Laboratories, Detroit, MI) for 48 h and maintained at 2°C through the course of the study as stock cultures. Serotypes of *C. botulinum* were verified on the basis of their toxin type based on neutralization by the homologous antitoxin and mouse bioassay *(15)*. In addition, *C. botulinum* strains were differentiated by cultural and biochemical characteristics.

Media

Reinforced clostridial medium (RCM) (Difco) and tryptic soy agar (TSA) (Difco) with and without added lysozyme (10 μg/ml) (Sigma Chemical Co., St. Louis, MO; 41,000 U/mg) were used as recovery media for heat-injured spores.

Preparation of spore suspension

For preparation of spores, 0.1 ml of spore stock culture, maintained in cooked meat medium, was inoculated into 10 ml of freshly prepared trypticase peptone glucose yeast extract (TPGY) (trypticase, BBL Microbiology Systems, Cockeysville, MD; all others, Difco) broth contained in screw-capped tubes and incubated at 28°C for 48 h. The contents of the culture tube were transferred into 90 ml of TPGY broth in a 100-ml Wheaton bottle and incubated at 28°C for 24 h. The 100-ml culture was transferred into 1 l of TPGY in screw-capped bottles and incubated at 28°C for 7 days. Spores were harvested by centrifugation at 4,000 × *g* for 10 min and then washed three times in sterile distilled water by repeated suspension and centrifugation at 4,000 × *g* for 5 min. Viable spore counts were performed by spiral plating (Spiral Biotech, Bethesda, MD; Model D) appropriate dilutions (in 0.1% peptone water), in duplicate, on RCM followed by incubation of plates for 48 h at 28°C in anaerobic jars (BBL GasPak Anaerobic Systems, Beckton Dickinson, Cockeysville, MD). The spore suspensions were adjusted to approximately 7 to 8 $\log_{10}$ spores per ml and stored at 2°C. Vegetative cells were not considered to be present because they were unlikely to survive in this method of spore preparation, which involved aeration during harvesting, repeated washing and suspending in distilled water, and centrifugation *(13, 26, 39)*.

Preparation of phosphate buffer and turkey slurry

Phosphate buffer (0.1 M, pH 7) was prepared according to Gomori *(16)*. The buffer was sterilized by autoclaving at 121°C for 15 min and stored at 4°C. Ground turkey was purchased from a local retail market. A known weight of turkey was aseptically transferred to a sterile Waring Blender and mixed with an equal volume of sterile distilled water by blending for 2 min to form a homogeneous slurry.

Thermal inactivation and enumeration

Before use, 10-ml portions of spore suspensions were sonicated (amps 2.5) at room temperature (Branson 3200, Bransonic Ultrasonics Corporation, Danbury, CT) for 20 min to break up spore clumps. This was done to help prevent survivor-curve tailing, known to result from spore clumping *(7)*. Buffer and turkey slurry were inoculated with heat-shocked (60°C, 10 min) nonproteolytic type B or type E spores to obtain an initial count of about 7 $\log_{10}$ spores per ml. Inoculated samples were blended or mixed with a vortex mixer to ensure uniform distribution of spores. The mixture was dispensed in 9-ml portions into sterile 17 by 60 mm screw-capped vials. Negative controls included vials containing uninoculated buffer or turkey slurry. Each vial was sealed with a sterile lid fitted with a rubber septum and submerged in a temperature-controlled water bath (Exacal, Model Ex-251HT, NESLAB Instruments, Inc., Newington, NH) stabilized at 70, 75, 80, 85, or 90°C. The temperature was continuously monitored by two copper-constantan thermocouples inserted at the center of two uninoculated vials. The thermocouple readings were measured and recorded using a Keithyl-Metrabyte data logger Model DDL 4100 (Tauton, MA) connected to a microcomputer. The thermocouple signal was sampled every second, and the two readings were averaged to determine the vial internal temperature. The "come-up" times to reach 70°C were approximately 2.5 min and 4 min for buffer and turkey, respectively; for 90°C, the times were < 1 min and 2 min. Come-up times were included as part of the total heating time when these were used to calculate the D-values. Once the mixture reached the target temperature, two vials were removed and designated as time 0. Thereafter, two vials for each replicate were removed at designated time intervals; sampling frequency was based on the heating temperature. After heating, vials were plunged into a crushed-ice bath. The surviving population was determined by enumerative spiral plating onto agar dishes containing RCM and TSA, with and without lysozyme, followed by anaerobic incubation at 28°C for 6 days. For each replicate experiment, an average surviving spore count of four platings of each sampling point was used to determine the D-values. The study was divided into two parts, with each part (in duplicate) replicated twice. The first part of the study determined the heat resistance of nonproteolytic spores in phosphate buffer. After D-values were determined in phosphate buffer at 70, 75, 80, 85, and 90°C, the second part of the experiment was carried out to determine the heat resistance of one strain of each type (most heat-resistant type B and randomly selected type E) in turkey slurry.

Calculation of D-values and z-values

To determine the D-values (negative reciprocal of the slope), a survival equation was fitted to the experimental data using a Gauss-Newton curve fitting program (ABACUS Software Program, ERRC, USDA, Philadelphia, PA). This equation, given below, was developed by Whiting *(40)* and was derived from the logistic-based equation of Kamau et al. *(22)*.

$$\text{Log}\frac{N}{N_0} = \text{Log}\left[\frac{F1\ (1 + e^{-b1*t1})}{(1 + e^{b1(t\ -\ t1)})} + \frac{(1\ -\ F1)\ (1 + e^{-b2*t1})}{(1 + e^{b2(t\ -\ t1)})}\right]$$

where

b1: maximum specific death rate of major population;
b2: maximum specific death rate of subpopulation;
F1: fraction of initial population in major population;
(1 - F1): F2, fraction of population in subpopulation;
t1: shoulder or lag period;
t: time;
N: population (log CFU ml^{-1}) surviving at time t;
N_0: initial population (log CFU ml^{-1}) at time 0; and
D-value: 2.3/b for each population.

The z-values were estimated by computing the linear regression *(27)* of $\log_{10}$ D-values versus heating temperatures using Lotus 1-2-3 Software. The z-value was estimated by taking the absolute value of the inverse slope.

RESULTS

Heated nonproteolytic *C. botulinum* spores exhibited lag periods or shoulders, where the population remained constant, followed by a linear decline. Also, a subpopulation of more persistent spores was observed that declined at a slower rate. Better recovery of surviving spores was obtained on RCM supplemented with lysozyme (Tables 1 and 2). Thus, higher "apparent" D-values were obtained when RCM with lysozyme was used. The D-values for *C. botulinum* type E spores in buffer ranged from 30.63 min at 70°C to 0.80 min at 85°C for strain Alaska; 44.58 to 1.03 min for strain Saratoga; and from 25.50 min to 0.68 min for strain Whitefish (data not shown). The D-value for the most heat-resistant type E strain, Saratoga, was 4.51 min at 80°C (Table 1).

The D-values of *C. botulinum* nonproteolytic type B strains 17B and CBW 25 in buffer at 70, 75, 80, and 85°C and strain KAP B5 at 75, 80, 85, and 90°C were determined. The D-values at 70°C were 24.16 min for strain 17B and 46.03 min for strain CBW 25. The D-value for strain KAP B5 was too long and was not determined at 70°C. As would be expected, the D-values declined as the temperature increased. The D-values at 75°C ranged from 5.45 min for strain 17B to 27.42 min for strain KAP B5 (data not shown); the D-values at 80°C ranged from 3.22 min for strain 17B to 4.31 min for strain KAP B5 (Table 2); and the D-values at 85°C ranged from 0.41 min for strain 17B to 0.89 for strain KAP B5 (data not shown). The D-value at 90°C for strain KAP B5 was 0.46 min.

Turkey slurry offered protection compared to buffer at all temperatures. The D-values for *C. botulinum* type E strain Alaska were 51.89, 18.06, 13.37, and 1.18 min at 70, 75, 80, and 85°C, respectively (Table 3). The D-values in turkey slurry for *C. botulinum* nonproteolytic type B strain KAP B5 ranged from 32.53 min at 75°C to 0.80 min at 90°C (Table 3).

The z-values determined from D-values in phosphate buffer were 9.60, 9.88, and 10.08°C for type E strains Whitefish, Saratoga, and Alaska, respectively (Figure 1);

TABLE 1. *Heat resistance (expressed as D-values in min) for* C. botulinum *type E strains in phosphate buffer at 80 °C.*

Strain	Recovery Medium[a]	D-Values (min)[b]	RMS Error[c]
Alaska	RCM	3.91 ± 0.51	0.67
	RCM + L	4.35 ± 0.65	0.68
	TSA	2.60 ± 0.35	1.04
	TSA + L	4.31 ± 0.55	0.60
Saratoga	RCM	3.61 ± 0.40	0.23
	RCM + L	4.51 ± 0.68	0.90
	TSA	3.77 ± 0.30	0.15
	TSA + L	3.80 ± 0.64	0.84
Whitefish	RCM	0.39 ± 0.05	1.43
	RCM + L	1.03 ± 0.15	1.02
	TSA	0.39 ± 0.05	1.50
	TSA + L	0.98 ± 0.15	1.09

[a] Reinforced clostridial medium (RCM) and tryptic soy agar (TSA) with or without lysozyme (L).
[b] D-values shown are the means of two replicates, each performed in duplicate and expressed as mean ± asymptotic standard error.
[c] Root mean squares error.

TABLE 2. *Heat resistance (expressed as D-values in min) for nonproteolytic* C. botulinum *type B strains in phosphate buffer at 80°C.*

Strain	Recovery Medium[a]	D-Values (min)[b]	RMS Error[c]
17 B	RCM	0.60 ± 0.01	0.12
	RCM + L	3.22 ± 0.44	0.92
	TSA	0.67 ± 0.02	0.16
	TSA + L	3.22 ± 0.63	0.98
CBW 25	RCM	1.90 ± 0.19	0.16
	RCM + L	4.09 ± 0.83	0.57
	TSA	0.96 ± 0.17	0.80
	TSA + L	3.73 ± 0.66	0.55
KAP B5	RCM	1.53 ± 0.78	0.31
	RCM + L	4.31 ± 0.83	0.38
	TSA	2.25 ± 0.10	0.42
	TSA + L	2.53 ± 0.25	0.43

[a] Reinforced clostridial medium (RCM) and tryptic soy agar (TSA) with or without lysozyme (L).
[b] D-values shown are the means of two replicates, each performed in duplicate and expressed as mean ± asymptotic standard error.
[c] Root mean squares error.

TABLE 3. *D and z values for nonproteolytic* C. botulinum *type B and type E strains in turkey slurry between 70 and 90°C.*

Temperature	Type B, KAP B5		Type E, Alaska	
(°C)	D-Values (min)[a]	RMS Error[c]	D-Values (min)	RMS Error
70	ND[b]	–	51.89 ± 1.93	0.35
75	32.53 ± 1.23	0.24	18.06 ± 2.80	0.40
80	15.21 ± 1.80	0.17	13.37 ± 2.35	0.43
85	4.85 ± 0.93	0.20	1.18 ± 0.12	0.60
90	0.80 ± 0.12	0.23	ND	–
	z-Zalues (r)[d,e] (°C)			
	9.43 (0.98)		9.90 (0.94)	

[a] D-values shown are the means of two replicates, each performed in duplicate and expressed as mean ± asymptotic standard error.
[b] Not determined.
[c] Root mean squares error.
[d] D-values used to determine the z-values were the means of two replicates.
[e] z-values followed by correlation coefficients in parentheses were determined using simple linear regression.

the z-values were 8.35, 8.58, and 9.02°C for nonproteolytic type B strains KAP B5, CBW 25, and 17 B, respectively (Figure 2). The z-values calculated from D-values in turkey slurry were 9.43 and 9.90°C for type B strain KAP B5 and type E strain Alaska, respectively (Table 3).

DISCUSSION

The study examined and compared the thermal resistance of nonproteolytic type B and type E spores in phosphate buffer and in turkey slurry. Previous researchers have used water and various phosphate buffer concentrations as a heating menstruum for thermal-inactivation studies *(4, 19, 32)*. The thermal-inactivation rates obtained in this study were, in general, consistent with those reported in the literature. Roberts et al. *(32)* used water as the heating menstruum for type E spores and reported D-values at 80°C of 1.25 min. In a study by Ito et al. *(19)*, D-values ranged from 0.77 to 1.95 min for a cocktail of four strains of type E in 0.02 to 0.03 M phosphate buffer (pH 7.0). Several workers reported that the D-values at 82.2°C for type E spores ranged from 0.15 to greater than 4.90 min depending upon the strain and heating menstruum *(3, 24, 36, 38)*. Scott and Bernard *(36)* investigated the

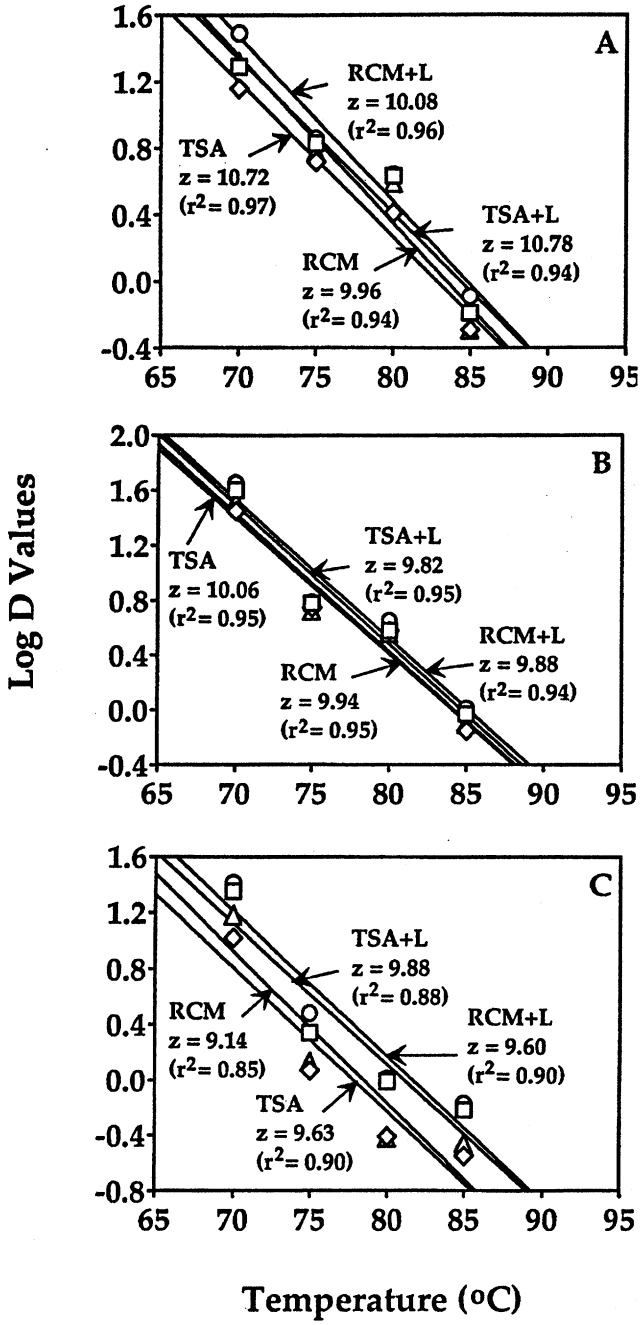

Figure 1. *Thermal-death-time curves (z-values) for* C. botulinum *type E strains Alaska (A), Saratoga (B), and Whitefish (C) over the temperature range 70 to 85°C. D-values, obtained in phosphate buffer, used to determine the z-values, were the means of two replicates and were obtained based on survivors on four media: reinforced clostridial medium (RCM) and tryptic soy agar (TSA), both with or without lysozyme (L).*

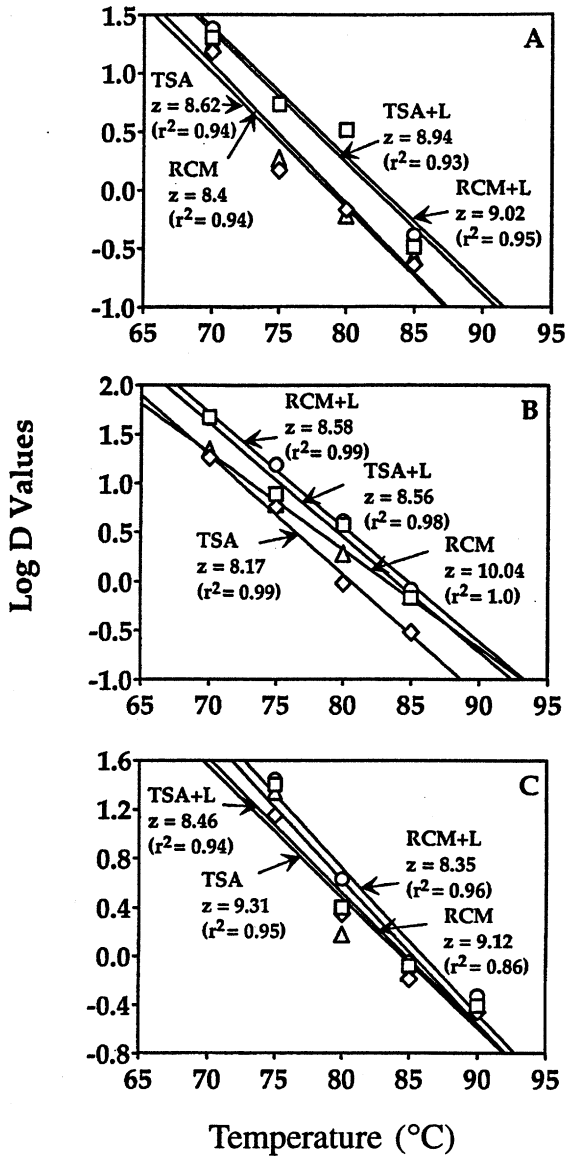

Figure 2. *Thermal-death-time curves (z-values) for* C. botulinum *nonproteolytic type B strains 17 B (A), CBW 25 (B), and KAP B5 (C) over the temperature range 70 to 90°C. D-values, obtained in phosphate buffer, used to determine the z-values were the means of two replicates and were obtained based on survivors on four media: reinforced clostridial medium (RCM) and tryptic soy agar (TSA), both with or without lysozyme (L).*

heat resistance of several nonproteolytic type B strains in phosphate buffer and reported D-values at 82.2°C ranging from 1.49 to 32.30 min, depending upon the strain. However, in their studies, the higher heat resistance values were not obtained consistently. Interestingly, the heat resistance of the nonproteolytic type B spores increased or decreased after storage for several months.

Slight differences in D-values obtained in our study and those reported by previous workers may be attributed to several factors. Previous researchers used the fraction-negative method, with recovery in an optimal medium after heat exposure for various times. Survival is determined by observing growth or no growth after incubation. In such studies, the D-values reported may be influenced by a few heat-resistant spores, since the shape of the curve relating the heating time to the number of survivors is not known. Moreover, sporulation media and the temperature used for spore preparation, heating menstruum, recovery conditions (including the composition and pH of the medium), the presence of inhibitors, temperature and time of incubation, and above all, the presence or absence of lysozyme in the recovery medium affect the calculated spore heat resistance *(14, 33)*. Recovery of thermally injured spores is significantly increased when the concentration of solids used in the recovery medium is lower than currently recommended levels *(21, 41)*. Scott and Bernard *(35)* suggested that there may be significant variations among strains, cultures of the same strain, and the reported D-values by different investigators within the same strain.

We demonstrated and confirmed previous studies that lysozyme in the recovery medium increases the recovery of the heated spores, thereby increasing the measured or apparent heat resistance *(28, 29, 36)*. In a study by Sebald et al. *(37)*, when *C. botulinum* type E spores were heated in phosphate buffer for 10 min at 80°C, surviving spores were able to form colonies on a medium containing lysozyme, but not in its absence. Alderton et al. *(2)* reported an estimated D-value of 1.30 min when type E spores were heated in phosphate buffer at 79.5°C and recovered on a medium without lysozyme. In the same study when the recovery medium contained lysozyme, the heating temperature had to be raised to obtain measurable spore destruction; the D-values were 13.50 and 3.80 min at 90.5°C and 93.3°C, respectively. Peck et al. *(28)* reported that the presence of lysozyme in the recovery media resulted in biphasic survivor curves, indicating heat-sensitive and heat-resistant population subfractions. Duncan et al. *(11)* suggested that the heat alteration of the spore results in inactivation of the cortex lytic enzyme system, i.e., the system responsible for cortical degradation during germination. Lysozyme in the plating medium can replace the thermally inactivated spore-germination enzymes *(36)*. The lysozyme is able to permeate the spore coat and degrade the cortex, allowing core hydration and consequently, spore germination *(17)*.

The increased thermal resistance of the spores in turkey slurry compared to buffer may be attributed to differences in composition (more solids in turkey slurry) between the substrates. Jay *(20)* indicated that food carbohydrates, fats, proteins, salt, etc., confer protection to bacterial spores against heat. Thus, it would be misleading to predict the thermal-death-time values in foods from data obtained in buffer. The higher D-values obtained in turkey slurry compared to buffer are consistent with those reported in the literature for foodstuffs. D-values at 80°C of 10.50 and 4.50 min for type E spores were obtained in tuna and sardines, respectively, whereas D-values at 80°C of 1.10 and 1.40 min were obtained in 0.017 M and 0.067 M phosphate buffers, respectively *(4)*. In another study, Lynt et al. *(25)* indicated that the heat resistance of nonproteolytic *C. botulinum* type F is similar to that of type E spores and reported that the D-values of type F spores were two- to fourfold less in 0.067 M phosphate buffer than in crabmeat. The data presented in Table 3 can be used to predict the time required at specified temperatures to achieve a certain number of log-cycle reductions of the spores when heated in turkey.

Thermal-death-time curves (z-values) for *C. botulinum* nonproteolytic type B and type E strains were constructed by plotting $\log_{10}$ D-values versus heating temperatures. The z-values were calculated from the slope of the line of best fit using linear regression. Our study indicates that smaller changes in temperature are required to cause a 90% reduction in the D-value, in buffer, in the case of nonproteolytic type B strains compared to type E strains. Similar results were obtained in turkey slurry in which type B strain KAP B5 and type E strain Alaska were evaluated.

Nonproteolytic *C. botulinum* type B and type E strains are potentially hazardous contaminants in food products which have been pasteurized and stored under refrigeration. A mild heat treatment given to such products could serve as an activation step if it were not lethal to the spores. Based on the thermal-death-time values determined in this study, contaminated turkey should be heated to an internal temperature of 80°C for at least 91.3 min; this is based on the argument that thermal treatments must be designed to achieve a 6-D process for nonproteolytic *C. botulinum* spores. Further studies are under way to develop predictive models from combinations of individually sublethal growth barriers as an approach to assess the safety of minimally processed foods. The intrinsic properties of the food products, primarily pH, sodium chloride, antimicrobials, etc., are known to affect the heat sensitivity of spores and the ability of surviving spores to grow out in pasteurized, refrigerated foods. Once the effects and interactions of these multiple food formulations in turkey are assessed and a mathematical model for spore inactivation is developed, food processors would be able to formulate foods and design a thermal process that provides a safe product while maintaining the desirable organoleptic attributes of the food. The assurance of safety from psychrotrophic *C. botulinum* spores is a key factor in the success of the new generation of refrigerated food products.

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